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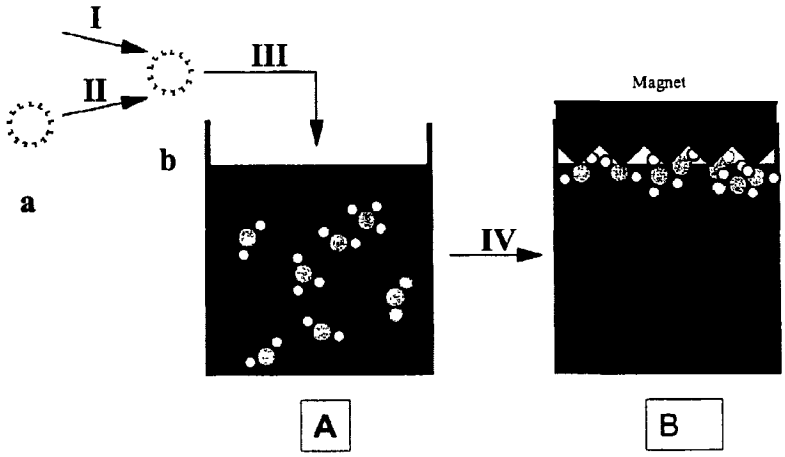
(54) Title: MAGNETIC NANOPARTICLES FOR BIOSEPARATION APPLICATION AND METHODS FOR PRODUCING SAME



(57) Abstract: This invention relates to the controlled crystallization of magnetite to form superparamagnetic nanoparticles. The particles are highly crystalline magnetite in the 10 to 250 nm, 10 to 200, 20-200 and preferably 50 to 150 nm diameter range, exhibiting superparamagnetic characteristics with a saturation magnetization of 62 emu/g. The particles are stabilized with a coating such as dextran. These particles have potential applications in biological cell separations, drug delivery and nondestructive clinical diagnosis.

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TITLE

Magnetic Nanoparticles for Bioseparation Application and Methods for Producing Same

PRIORITY APPLICATION

This application claims the benefit of priority from United States Provisional Patent Application No. 60/302,379, filed July 3, 2001, entitled " Engineering of Magnetic Nano Particles for Bioseparation".

FIELD OF THE INVENTION

This invention relates to the field of magnetic, preferably paramagnetic or superparamagnetic, and methods and uses thereof, including for bioseparation applications.

BACKGROUND OF THE INVENTION

Particles in the 1-200 nm diameter size range, considered as nanoparticles often exhibit unique optical, electrical, chemical, structural and/or magnetic properties. For example, magnetite particles greater than 1 μm in diameter are ferromagnetic, exhibiting a large magnetization hysteresis and a strong remnant magnetic induction (M_r) whereas magnetite particles less than 200 nm are superparamagnetic with little magnetization hysteresis while maintaining a strong saturation magnetization (M_s) (Leslie-Pelecky and Rieke, 1996). This class of paramagnetic nanoparticles have a wide range of potential applications, including information storage, color imaging, bioprocessing, diagnostic microbiology, biosensors, drug delivery, magnetic refrigeration, ferrofluids and magnetic switchers. Applications using magnetic nanoparticles as magnetic carriers or magnetic tags have also been described (Williams, 1992). The objective of magnetic carrier technology is to confer magnetic properties to a naturally non-magnetic target so that the target can be separated from the non-target entities using magnetic separators. This is particularly useful for separations in multiphase systems of a complex nature.

Figure 1 summarises schematically the processes involved in a typical magnetic carrier technology application. In order to make a target magnetic, the target has to be recognised by and attached to a magnetic carrier. To accomplish this often requires the paramagnetic particles to be functionalized, usually by derivatizing a surface or putting a specific ligand on the particle (step I), or by in situ formation of a nanosize magnetic core in a structured template, e.g. micells, vesicles, porous silica or polymer matrix (step II). The particles prepared as such, termed as magnetic carrier (b), are added to a biological system containing unwanted targets, such as infected blood cells (white circles in c). The selective attachment of infected cells to the magnetic carriers is accomplished through biomolecular recognition, conferring the magnetic property to the infected cells only (step III). By exposing the resultant biological system to an external magnetic field, the labelled cells are directed to a desired location (the top of the system in this case), where they can be removed readily without disturbing the system (step IV). A principal attraction of using magnetic carrier technology in clinical applications is its minimised side effect, compared with traditional treatments, such as chemotherapy which destroys healthy cells as well. (Bacri et al., 1997; Mitsumori et al., 1996).

United States Patent 4,452,773 to Molday, issued June 5, 1984, describes the production of colloidal size ferromagnetic iron oxide (Fe_3O_4) coated with a water-soluble polysaccharide (such as dextran) or reactive derivative thereof and to a process of preparing same. In the method an alkali solution is added to a solution of a ferrous and ferric salt. The particles formed are then separated by centrifugation and gel filtration. The particles in the invention have a diameter of about 100-700 Angstrom, more particularly about 300-400 Angstrom.

Liberti, US 5,512,332 issued April 30, 1996, describes the production of magnetic particles of small size (maximum particle size generally below 0.2 μm) with a stabilizing (preferably biochemically or biologically active) coating. This patent teaches using a starting material such as magnetite ($FeO.Fe_2O_3$) that is in the form of a crystal agglomerate (larger sized particles formed by

adding an alkali solution to a Magnetite solution). These crystal agglomerates can then be treated in a number of ways to disrupt or subdivide the magnetic particulate starting material. Coating material can either be added contemporaneously with the starting material or alternatively, can be added after the disruption has been accomplished, but before the small particulate begins to re-aggregate. The particles are separated using high gradient magnetic separation (HGMS).

Liberti, US 5,597,531 issued January 28, 1997. This patent teaches a procedure for a resuspendable (colloidal) bioactive product. However, this re-suspendable product is produced by the same method as in the Liberti, US 5,512,332 patent noted above.

However, the magnetic-dextran particles and processes for producing same, described in the above-noted patents all have deficiencies. For biological cell separation using magnetic carrier technology (Kronick and Gilpin, 1986) it is important to avoid mechanical instability of the particle suspension due to aggregation and sedimentation under gravity and to minimize the effect of the particles on cell function and on assay techniques. To avoid mechanical instability the particles must be sufficiently small (in the nano size range) that thermal energy and surface forces prevail. It is also important to avoid magnetic flocculation, a phenomenon where magnetic particles agglomerate by magnetic forces. With particles less than 200 nm, the remnant magnetic induction is negligible so that the permanent magnetization is avoided and magnetic flocculation minimized. However, as the particles become increasingly smaller, the magnetic force on a magnetic particle in an external magnetic field becomes progressively weaker with all other things being equal. As a result, subsequent magnetic separation becomes extremely difficult unless a high gradient magnetic separator (HGMS) is used, which can be unattractive in many biological applications where a disposable column must then be used to avoid cross-contamination.

There is a need for a process to make magnetic particles of an optimal size range that maximizes magnetic forces under the applied external

magnetic field while exhibiting superparamagnetic characteristics and colloidal and chemical stability.

SUMMARY OF THE INVENTION

The present invention provides an improved method for synthesizing superparamagnetic nanoparticles. These nanoparticles can be used in a variety of applications, for instance environmental, biological, chemical applications. In one embodiment, these nanoparticles are particularly useful in biological applications. In another embodiment, the nanoparticles are useful in tagging, monitoring chemical or biochemical reactions, monitoring the presence, production, synthesis, break down of certain substances, isolation and/or removal of substances from a sample, including purification, detoxifciation, diagnostic, treatment applications. The invention also provides the resultant nanoparticles and methods and uses thereof.

The present invention provides a method of synthesizing paramagnetic, preferably superparamagnetic magnetite particles. The method comprises:

a) crystallizing magnetite to form superparamagnetic particles under controlled crystallization conditions by adding a solution of ferric and ferrous ions to an alkaline solution, thereby limiting the duration of ferric and ferrous ion supersaturation and the extent of magnetite crystal growth;

b) controlling aggregation of said magnetite particles to control the size distribution of said particles and;

c) coating said particles with a coating substance to generate non-aggregating particles, thereby stabilizing the particle suspension.

In one embodiment, all or part of the method, such as steps (a) and/or (b) is performed under conditions that inhibit the oxidation of Fe2+ to Fe3+ iron, such as under redox conditions. In one embodiment, such redox conditions can be obtained by nitrogen sparging or chemical equivalent

thereof, vacuum or other method known in the art, to obtain an environment of reduced or no oxygen.

In another embodiment controlling aggregation comprises permitting or reducing or inhibiting aggregation as desired. In a preferred embodiment, controlling aggregation means preventing aggregation to the degree desired. Where it is understood that preventing or inhibiting aggregation means that aggregation is prevented or inhibited to at least some degree to control crystal growth and particle size, but with regard to a whole sample a certain degree of aggregation may exist. A person skilled in the art upon reading this description would understand the degree of prevention within a particular sample that would be beneficial to produce the desired superparamagnetic nanoparticles of the invention and the amount thereof and factors that can be adjusted to control aggregation.

In another embdodiment, aggregation of particles is controlled by physical means, for instance by sonication, such as ultrasonication. Sometimes an untrasonication prevents aggregation.

In yet another embodiment, of the method of the ratio of ferric to ferrous ions is about 1.5: 1– 2.5:1, preferably about 2:1.

In one embodiment, the solution of ferric and ferrous ions has a pH of about 2.5.

In another embodiment the alkaline solution is a sodium hydroxide solution, for example a 1.5M sodium hydroxide solution.

In one embodiment, the coating substance is a water-soluble substance that is suitable for inhibiting particle aggregation and that can be coupled to a desired target molecule directly or indirectly. In another embodiment, the water-soluble substance is a water-soluble polysaccharide for example dextran, dextrin, starch, guar, gelatin, agar, sucrose or dextrose.

In an embodiment the coating substance is dextran, such as dextran with a molecular weight of between 4,000 to 2,000,000, preferably, 4,000 – 70,000, more preferably about 35,000 – 45,000, or about 40,000.

In yet another embodiment, the coating substance is 0.25M dextran solution.

In one embodiment, the coating substance is BSA, antibodies, natural or synthetic polymers, protein, polypeptides or nucleic acid polymers.

The magnetite nanoparticles, preferably supermagnetic particles produced by the method of any of the embodiments of the present invention is also intended to be encompassed within the scope of the invention.

In another aspect, the invention provides a method of rendering a biological target magnetizable comprising, functionalizing the magnetite nanoparticles produced by the method of the invention to enable them it to couple with the desired target, such as a biological target and then exposing the target to the functionalized magnetite nanoparticles to form a nano particle-target complex. In one embodiment, the magnetite nanoparticles of the invention are functionalized by derivatizing the surface of the particles and/or by putting a specific ligand on the particle. In one embodiment, it is the coating substance of the particle that is functionalized. In another embodiment, the nanoparticles are functionalized by an anti-coating substance antibody, such as a dextran antibody. In yet another embodiment, the biological targets are cells, protein, nucleic acid molecules, such as DNA or RNA. In another aspect, the invention the method includes recovering the nano particle-target complex from a sample by magnetic separation, for instance by exposing the sample to an external magnetic field gradient.

In a preferred embodiment, the present invention provides an integrated fabrication route for synthesizing superparamagnetic magnetite nanoparticles stabilized by water soluble dextran molecules. The adsorbed dextran molecules on the synthesized magnetite particles exhibit specific binding with anti-dextran antibody complexes which in turn bind with antigen on target cells, making these cells amenable for magnetic separations.

Other features and advantages of the present invention will become apparent from the following detailed description. However, it should be understood that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and

scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in reference to the figures which are for illustrative purposes only and not meant to be restrictive of the scope of the invention.

Figure 1 is a schematic diagram illustrating the concept of magnetic carrier technology in biological cell separations.

Figure 2 is a graph of concentration versus time illustrating the principle for wet chemical synthesis of nanosize solid particles (Hunter, 1993).

Figure 3 illustrates the X-ray diffraction patterns of the synthesized solid particles (the vertical lines represent the diffraction peak positions of the standard magnetite with the line height showing the relative intensity of the diffraction peaks.).

Figure 4 illustrates the improvement in the signal to noise ratio of the X-ray diffraction patterns of the synthesized solid particles when the redox environment is controlled (with and without nitrogen sparging). The results suggest that the particles formed with redox control have a greater crystalinity.

Figure 5 illustrates the magnetization of the synthesized solid particles with (closed circles) and without (open circles) nitrogenation by adding a ferrous/ferric ion solution to an alkaline solution at pH 12.5, showing a strong magnetism with a clear superparamagnetic characteristic for particles prepared with redox control by nitrogenation.

Figures 6 is a graph illustrating particle size distribution of the synthesized magnetic particles (adding ferrous/ferric ion solution to an alkaline solution at pH 12.5).

Figure 7 is transmission electron micrographs of the synthesized particles showing: A) morphology; and B) electron diffraction pattern.

Figure 8 illustrates the role of supersaturation level control in synthesis of magnetic particles by adding ferrous/ferric to an alkaline solution at pH 12.5 vs the addition of NaOH solution to ferrous/ferric ion solution to a final pH of

12.5. The process of the present invention is illustrated with open circles and the prior art method is illustrated in closed circles.

Figure 9 demonstrates the role of ultrasonication strength in controlling the resultant average particle sizes. Ferrous/ferric solution was added to an alkaline solution at pH 12.5.

Figure 10 shows the effect of ultrasonication intensities on the magnetization of the resultant magnetic nanoparticles synthesized by adding ferrous/ferric to an alkaline solution at pH 12.5.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

The term "**about**" as used herein in reference to a value, includes any chemical or obvious equivalents or functional equivalents to the specified value. In one embodiment it refers to +/- 10% of the stated value.

The term "**Magnetite**" as used herein is an iron ore that is strongly attracted by a magnet. In one embodiment it has a general formula of Fe_3O_4 (preferably with a Fe^{2+} to Fe^{3+} ratio of about 1:1.5 to about 1:2.5 , preferably about 1:2) or chemical equivalents thereof such as $(Fe^{2+}, M)O\ Fe^{3+}{}_2O_3$ where M can be, but not limited to Zn, Co, Ni, Mn, Cr. These examples are not meant to limit the scope of this invention. For instance, the Fe^{2+} to Fe^{3+} ratio includes any ratio that permits the formation of superparamagnetic nanoparticles in accordance with the method of the present invention.

The term "**paramagnetic**" as used herein refers to a class of substances that when placed in a magnetic field are magnetized parallel to the line of force in the field and proportional to the intensity of the field.

The term " **superparamagnetic**" as used herein refers to a class of substances that have a similar magnetism as ferromagnetic materials in the external magnetic field, but does not have a remnant magnetization after removal of the external magnetization field.

The term "**magnetization hysteresis**" as used herein refers to a lagging effect behind its cause, for instance a magnetic body subject to a varying

force. This can result in an internal magnetic field in the material that may show long term drifts.

The term "**Water soluble**" as used herein means those substances, (such as polysaccharides, or more specifically dextran,) that will remain in aqueous solution as individual molecules at ambient temperature (e.g. about but not limited to15-25 degrees Celcius.

The term "**Stablized**" as used herein in reference to paramagenitc or supermagnetic particles refers to the stability of the resulting particles in solution after the reagents have been mixed. Stabilization means that the particles are stable without aggregation or settling out of solution. Further that there is no change in the chemical composition of the magnetic particles under specified conditions over a particular period of time.

The term "**particle size distribution**" as used herein means the particle size range and the proportion of the magnetic nanoparticles at a particular particle size or sizes.

The term "**redox environment or conditions**" as used herein refers to an environment that has reduced oxygen levels as compared to ambient conditions, preferably that is substantially free of oxygen. It refers to an environment where the net oxidation of iron in the divalent state to the trivalent state is minimized or preferably prevented. For example, such an environment can be obtained by nitrogen sparging (e.g. to displace oxygen) or by other means known in the art (vacuum, sparging with other inert gases e.g. argon.

The term "**nucleation**" in reference to crystallization of magnetite as used herein means the formation of a core site of crystalline Fe_3O_4. Each nucleus can spawn a crystal particle. In one embodiment it refers to the formation of Fe_3O_4 crystals that are formed from the addition of the iron solution to the sodium hydroxide solution.

The term "**polysaccharide**" as used herein refers to any water soluble polysaccharide, including, but not limited to dextran, dextrin, starch, guar, gelatin, agar, sucrose and dextrose.

The term "**dextran**" as used herein refers to but not limited to neutral or weakly charged dextran with a molecular weight range of about 4,000 to 2,000,000, or 4,000 to 70,000 or, preferably from about 35,000 to 40,000. Examples of such dextran include Dextran T40 from Amersham Pharmacia Biotech.

The term "**nanoparticle**" as used herein refers to a particle having a size in the order of 10^{-9}m range. In one embodiment the nanoparticles of the present invention are in the range from about10 to 250nm, 20 to 250nm, 30 to 250nm, 10 to 200nm, preferably about 20 to 200nm, 30 to 200nm, or 50 to 150 nm, with a mean and/or average size of the nanoparticles preferably being 150nm.

The term "**saturation magnetization**" also known as maximum magnetization, refers to a point when all of the magnetic moments in a sample are aligned.

The term "**controlled crystallization**" as used herein means a process wherein the growth or size of crystals can be controlled (increased, decreased or remain the same) during the course of a reaction. Controlled crystallization can be effected through a number of methods in this invention, including but not limited to one or more of the following: sonication intensity, pH of alkaline solution, concentration of iron solution and ratio of ferric to ferrous ions. In a preferred embodiment of this invention, crystallization is controlled by using sonication at an instrument output of 35%, adding the ferric/ferrous solution to the sodium hydroxide solution with a preferred ratio of two ferric ions to one ferrous ion or functional chemical or obvious equivalents thereof.

DESCRIPTION

A number of approaches have been developed to synthesize nanoparticles (Chow and Gonsalves, 1996). The examples include: gas phase atomization (aero sols), evaporation/condensation, bulk wet chemical synthesis with sonochemistry or in templated microreactors such as porous matrix (zeolites), microemulsions, vesicles or micelles. The approach of the present invention is to control nucleation and particle growth by controlling the crystallization chemistry. Based on the La Mer diagram for inorganic synthesis

shown in Figure 2 [principal for wet chemical synthesis of nanosize solid particles, Hunter, 1993], a crystallization process represented by the solid curve would generate a large number of nuclei by creating a local supersaturation level while controlling the level of crystal growth by resultant sparse concentration conditions of the bulk solution.

In a preferred embodiment this is achieved by adding the iron solution to an alkali solution under conditions that control (e.g. inhibit or minimize) aggregation between magnetic nuclei and/or crystal particles (such as by ultrasonication). This addition method generates a large number of nuclei by creating a local supersaturation level while controlling the level of crystal growth by resultant sparse concentration conditions of the bulk alkaline solution.

The particles are then stabilized by coating them with a coating substance that inhibits aggregation between particles and preferably maintains the resultant particle size distribution. The coating substance is preferably a biocompatible substance that can be used in bioseparation, biotagging or biomonitoring applications *in vivo* and/or *in vitro*. The coating substance is also preferably selected for its ability to be functionalized to couple with a target substance. This can be done by coupling the coating substance with another substance such as substances that can both couple with the coating substance and a specific desired target molecule. For instance the coating substance can be dextran, the other substance can be an anti-dextran antibody that can bind dextran and also has specificity to a desired target such as a particular cell surface receptor, cell type, protein, nucleic acid molecule or antigen so it can form a magnetic nanoparticle – target complex that can be separated from non-magnetic particles by application of a magnetic field, such as an external magnetic field. The coating substance can also be derivatized to couple with the target substance, preferably a biological target.

METHOD OF PRODUCING PARAMAGNETIC NANOPARTICLES

The present invention provides a method to synthesize paramagnetic, preferably superparamagnetic nanoparticles comprising controlled crystallization of magnetite (Fe_3O_4) as well as other chemical equivalent compounds such as (Fe^{2+}, M) $Fe^{3+}{}_2O_4$, where M can be,but not limited to Zn, Co, Ni, Mn, Cr, under controlled aggregation conditions and stabilization of the particles by coating with a polysaccharide, preferably a water soluble polysaccharide, such as dextran with a preferable molecular weight between 35,000 to 45,000, but not limited to that range. Other ranges can work, for instance 4,000 to 2,000,000. Other water soluble polysaccharides covered by this invention include but are not limited: dextrin, starch, guar, gelatin, agar, sucrose and dextrose.

A solid product in the 30 to 250 nm diameter range was produced by adding a concentrated iron solution to an alkaline solution at pH above 8, preferably at pH 12.5 under ultrasonication to prevent particle aggregation. The particles were stabilized with dextran. The synthesized particles were identified with x-ray diffraction as composed largely of magnetite and to be superparamagnetic with a saturation magnetization of about 62 emu/g as determined with a Quantum Design PPMS magnetometer. Transmission electron micrographs showed that the particles were rounded without noticeably sharp edges. These particles exhibited excellent stability and showed satisfactory blood cell recovery with magnetic separations. This method is an improvement over previous methods.

In a preferred embodiment of the method, the magnetic nanoparticles of the invention are synthesized under a well controlled redox environment in one step (for instance without requiring subsequent frationation) with controlled particle size and crystallinity. The redox environment controls the oxidation state of iron species and preserves the preferable ratios of iron between two different oxidation states (e.g. ferric to ferrous ratio). The preferred redox environment is one that is of a reduced oxygen or other oxidizing compound, content in aqueous solutions (compared to ambient conditions), preferably free of molecular oxygen, which can be accomplished

by sparging with an inert gas such as nitrogen, but may also be done with argon. As the crystallization of the nanoparticles can be strictly controlled, the particle size distribution of the resultant particles is such that there is no need for separation or fractionation when the reaction finishes because the particles are all in a suitable size range. In one embodiment the suitable size range is between 10 – 250 nm, 10 to 200 nm, 20 to 250 nm, 20 to 200 nm, 30 to 250 nm, 30 to 200 nm, or 50 to 150 nm. In another embodiment the mean and/or average particle size is about 150nm. This one-step synthesis avoids time-consuming and poorly reproducible disruption of solid magnetite particles. By controlling particle size and crystalinity, the need for fractionation of the coated particles on the basis of size or magnetization is reduced or eliminated.

More specifically, the present invention in one embodiment provides a method of synthesizing paramagnetic, preferably superparamagnetic magnetite particles comprising:

(a) controlling the crystallization to form paramagnetic, preferably superparamagnetic particles by adding a solution of ferric and ferrous ions , preferably in a ratio of about 1.5:1 – 2.5:1, more preferably about 2:1 and preferably having a pH of about 2.5, to an alkaline solution, such as a NaOH solution, such as a 1.5M NaOH solution. The alkaline solution in one embodiment has a pH of above about 8 and preferably about 12.5. The addition of the iron solution into the alkaline solution limits the duration of ferric and ferrous ion supersaturation and the extent of magnetite crystal growth. As stated it above, it generates a large number of nuclei by creating a local supersaturation level while controlling the level of crystal growth by resultant sparse concentration conditions of the bulk solution.

(b) controlling, preferably preventing aggregation of said magnetite particles to control the size distribution of said particles and;

(c) coating said particles with a coating substance to generate non-aggregating particles, thereby stabilizing the particle suspension.

In one embodiment the aggregation of particles is prevented by physical means, such as sonication, preferably ultrasonication.

In yet another embodiment, the method, at least preferably (a) and preferably (b), is conducted in a controlled redox environment to inhibit or prevent the oxidation of Fe^{2+} to Fe^{3+} ions. In yet another embodiment the redox environment is controlled by nitrogen sparging.

In one embodiment the coating substance is a polysaccharide, preferably a water soluble polysaccharide, such as dextran, for example a dextran having a molecular weigh of about 40,000. In another embodiment the dextran solution is a .25M dextran solution. In yet another embodiment the coating substance is Bovine Serum Albumin (BSA) antibodies, lipids, natural or synthetic polymers, protein, polypeptides or nucleic acid polymers.

THE PARAMAGNETIC NANOPARTICLES

The present invention provides paramagnetic, preferably super paramagnetic nanoparticles that can be functionalized to enable them to be used in bio applications such as bioprocessing, diagnostic microbiology, biosensors, drug delivery, magnetic refrigeration, ferrofluids and magnetic switchers, magnetic carrier technology, and applications in screening and purification or isolation. Preferably the nanoparticles are produced by the method of the present invention. The paramagnetic nanoparticles of the invention can be functionalized in many ways known to persons skilled in the art such as by derivatizing the surface of the particles by putting a specific ligand on the particle. In yet another embodiment the nanoparticles are functionalized by an anti-dextran antibody.

The paramagnetic particles of the invention are preferably iron oxides, such as magnetite, in a preferred ferrous to ferric iron ratio of about 1:2, but not limited to that ratio. Other embodiments include a ratio of ferrous to ferric

particles of about 1:1.5-2.5, doped with additive ions, such as but not limited to Zn, Co, Ni, Mn and Cr.

Functionalized paramagnetic or superparamagnetic nanoparticle means a nanoparticle that is stabilized or coated by a substance such as a polysaccharide, such as dextran. The stabilized paramagnetic nanoparticle can be further modified or can directly tag another molecule, preferably a biomolecule (e.g. a biological target) (e.g. a cell, a cell surface receptor, an antibody, a protein, nucleic acid molecules such as DNA or RNA) or other desired molecules in a preferably specific manner, to form a nanoparticle target complex. As such, the paramagnetic nanoparticle can confer magnetic properties to said non magnetic target substance that is desired to be tagged, monitored, distinguishable or separated from other molecules or substances. This can be done by using a coating substance that can be coupled to the magnetic nanoparticle of the invention directly to a desired target substance or through an intermediary substance or substances (e.g. a linker) that can both couple with the coating substance and the specific target substance. In one example the dextran is the coating substance, an anti-dextran antibody that also has specificity to the target substance couples to the dextran and then can be used to couple the target substance which can be subsequently removed, separated or detected using a magnetic field. The magnetic nanoparticles can also be functionalized by derivatizing the surface of the particles, putting a specific ligand on the particle through silanation or self-assembly by desired molecules, such as organic silanes, lipids, and surfactant molecules.

The invention also encompasses superparamagnetic nanoparticles coated (preferably coupled for instance, through chemical, physiochemical and/or hydrogen binding) with a suitable polysaccharide. Such polysaccharides include any water soluble polysaccharide, including, but not limited to dextran, dextrin, starch, guar, gelatin, agar, sucrose and dextrose. Preferred polysaccharides are dextran.

In one embodiment the polysaccharide is loaded on the superparamagnetic particles at a sutiable loading capacity such as greater

than 0.29g dextran/g solids, but the invention is not limited by such a loading capacity. A person skilled in the art can adjust the loading capacity to a desired level, for instance by adjusting the ratio of magnetite and coating substance, such as dextran used and the coating conditions.

In another embodiment the invention provides a paramagnetic, preferably superparamagnetic nanoparticles that have an average particle size of about 150 nm. In yet another embodiment, the size of the nanopartices produced is equally distributed around 150 nm, and preferably within a range of 20 – 250 nm but can be 10 – 250 nm, 30 – 250nm, 10 – 200 nm, 20 – 200 nm, 30 – 200 nm or 50 – 150 nm. In another embodiment, the product has a narrow particle size distribution in that the particles are primarily around 150 +/- 20 nm.

THE USE AND APPLICATIONS OF THE PARAMAGENTIC NANOPARTICLES

In one embodiment, the invention provides a paramagnetic nanoparticle that can confer magnetic properties to a substance or molecule of interest. It can act as a molecular tag or carrier. The substance or molecule of interest can then be separated from other molecules by application of a magnetic field, such as an external magnetic field gradient and separating the magnetic particles from the non-magnetic particles.

Thus the superparamagnetic nanoparticles of the invention can be used in monitoring the presence or amount of a desired substance in an assay, such as a bioassay, (e.g. environmental, diagnostic or other assay). The method can also be used to separate or remove a desired substance from other substances, such as to tag and remove cancerous or other cells or substances from a biological environment be it *in vitro* or *in vivo.* In *in vivo* applications, the magnetic nanoparticle should be biocompatible, in such that it is not harmful to a subject upon administration. In such a manner, the magnetic nanoparticles of the invention can be formed into a pharmaceutical composition and mixed with suitable pharmaceutically acceptable carriers or excipients, such as disclosed in *Remington's Pharmaceutical Sciences*, Mack

Publishing Company, Easton, Pa, USA, 1985. The nanoparticles can be used in the treatment or diagnosis of certain conditions such as in tagging, detecting and/or removing cancer cells for example from a sample or tissue. The invention can also be extended to detoxification and/or valuable recovery from domestic and industrial wastes.

The invention will be more fully understood by reference to the following examples. However, the examples are merely intended to illustrate embodiments of the invention and are not to be construed to limit the scope of the invention.

EXAMPLES

Materials

Analytical grade iron (III) sulfate tetrahydrate (97%) from ACROS, iron (II) sulfate heptahydrate (98+%) from ALDRICH and sodium hydroxide from ACP Chemicals were used as received. Dextran T40 with an average molecular weight 40,000 (Pharmacia Biotech, Sweden) was used as the stabilizer which adsorbs on nanoparticles for further binding with biomolecules. Millipore water was used in all the experiments.

Experimental procedures

Solution preparation: For each test, an iron solution at a ferric to ferrous molar ratio of 2:1 was freshly prepared by dissolving 5.90 g of iron(III) sulfate tetrahydrate and 3.47 g of iron(II) sulfate heptahydrate in 100 mL of deionized water in a 250 mL beaker, with a resultant pH of ca. 2.5. An alkaline solution was prepared by adding 6.0 g of sodium hydroxide in 100 mL of deionized water in a 500 mL beaker. To prepare a dextran solution, 5.0 g of dextran was added to 45mL of water in a 100 mL beaker.

Synthesis of stable magnetite nanoparticle suspensions: The prepared iron solution was added to the alkaline solution dropwise within 5 minutes under a strong ultrasonication environment with or without nitrogen sparging to control the redox environment. To avoid oxidation of ferrous to ferric ions

during the synthesis, sodium hydroxide aqueous solution was purged for 30 minutes with pure nitrogen through glass pipette tube prior to the addition of ferrous/ferric solutions. The nitrogen purge continued during the addition of the ferric/ferrous ion solutions and the dark precipitates were observed to form immediately during this period. This procedure ensures a local supersaturation level for nucleation, a condition shown in Figure 2 by the solid line, while maintaining a minimum bulk iron concentration to limit the duration of crystal growth represented by the dotted line. The resultant suspension was then transferred to the dextran solution at ambient or room temperature in the range of 15-25°C under agitation for 15 minutes, resulting in a stable dark suspension. The suspension was then centrifuged using a BECKMAN Ultracentrifuge (Allegra™ 64R) at 20,000-g for one hour. The supernatant was decanted and the solids were redispersed in 50 mL deionized water using an ultrasonic bath (FS30, Fisher Scientific). The redispersed particles were centrifuged again to decant the supernatant. This washing procedure was repeated for three times to ensure a complete removal of residual dextran in solution. The washed particles were then redispersed in deionized water.

Particle characterization: For the purpose of characterization, samples of particles prepared as described were dried in a vacuum oven at room temperature for at least 48 hours. The crystallinity of the particles was characterized using a Philips x-ray powder diffractometer (PW 1730, Philips). Magnetization characteristics of a precisely weighed volume of dry particles was determined at 300 K on a Quantum Design PPMS magnetometer (model 6000).

The size distribution of the synthesized particles was measured using a Zetasizer 3000 (Malvern Instrument Inc., Point Roberts, WA). The prepared suspension was diluted with deionized water and the measurement was performed at room temperature. To verify the particle size distribution measured with Zetasizer 3000 and to examine the morphology of the synthesized particles, a JEOL 2010 transmission electron microscope (TEM) was used to obtain TEM micrographs. In this case, the prepared suspension

was further diluted with deionized water and a holey carbon TEM grid was dipped into the diluted suspension. By carefully taking the grid out of the suspension, a fine drop of the suspension was retained on the grid. After evaporation of the water under ambient conditions, the specimen was transferred into TEM sample chamber and micrographs were taken in the conventional TEM mode.

Example I - *Crystallinity*

The X-ray diffraction pattern of the synthesized particles is shown in Figure 3. For comparison, the diffraction pattern of standard magnetite (Fe_3O_4) particles is also included in this figure as vertical lines with the line height representing the relative intensity of the corresponding peaks. The noisy background x-ray counts indicate the presence of a small amount of noncrystalline solid phases. This could be due to incomplete solid phase conversion of intermediate iron hydro-oxide complexes, although the exact nature of these noncrystalline solids is largely unknown. A near perfect match of peak positions and relative peak intensities with those in the standard diffraction pattern for magnetite confirms that the solid phase is mainly magnetite. It is evident from Figure 4 that the major solid phases formed are highly crystalline but that the crystillinity is greater for samples prepared with nitrogen sparging, in which case a significantly higher signal-to-noise ratio of the diffraction pattern was obtained.

Example 2 - *Magnetic Characteristics*

The magnetization characteristics of the formed particles is shown in Figure 5. For the reference, the saturation magnetization of standard maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4) is indicated by arrows in this figure. A saturation magnetization at 64 emu/g was obtained for the sample prepared with nitrogen sparging, suggesting a strong magnetization characteristics. Although this saturation magnetization value is higher than that for maghemite, it remains lower than the value for pure magnetite. Whether the lower saturation magnetization of the synthesized particles than

that of pure magnetite is due to incomplete solid phase conversion as suggested by x-ray diffraction pattern in Figure 3, remains to be established. A saturation magnetization at 25 emu/g was obtained for the sample prepared without nitrogen sparging as also seen in Figure 5, showing a much weaker magnetization characteristics than the sample prepared with sparging. This is likely due to the higher proportion of maghemite or other iron oxide present in the synthesized particles without nitrogen sparging, as seen in Figure 4. The magnetization loop of the formed solids, on the other hand, showed little magnetization hysteresis, confirming that the particles are paramagnetic, a feature highly desired in magnetic carrier technology. It is important to note that the magnetic properties of the resultant particles formed without nitrogenation is highly variable, while with nitrogenation, it is more reproducible, indicating an improved quality control of the synthesis.

Example 3 – *Particle Size*

Particle size is an important characteristic for biological applications. The size distribution of the synthesized particles is shown in Figure 6. It is clear that the particles synthesized as such exhibit a single modal particle size distribution peaking at 130 nm. About 90% of the particles are in the 80 to 230 nm diameter range. The particles in this diameter range are highly stable against gravitational sedimentation and "invisible" to biological cells.

The TEM micrograph in Figure 7A shows that the synthesized particles are indeed uniformly distributed around 150 nm diameter range, confirming qualitatively the determined particle size distribution using our Zetasizer. The particles are highly rounded without sharp edges. The electron diffraction pattern in Figure 7B clearly shows that the particles are highly crystalline, consistent with the observations from the XRD patterns.

To illustrate the role of the synthesis concept of controlling supersaturation level the synthesis was conducted at various final ferrous/ferric ion concentrations using two distinct procedures. First as in the previous arts, a highly concentrated alkaline solution (NaOH in the example) was added to the ferric/ferrous ion solution at a desired concentration. In an

embodiment of the present invention the ferric/ferrous ion solution is added to the alkaline solution, to minimize crystal growth and to better control crystal size resulting in a more uniform crystal size and narrower particle size distribution. Figure 8 shows the variation of the average particle sizes of the synthesized product with total iron ion concentration at a fixed 1:2 ferrous-to-ferric ratio. Compared to the procedures of the present invention (open circles), the average particle size with the conventional procedures (closed circles) is much greater and highly dependent on the initial total iron concentration. In the present invention, the average particle size is hardly dependent on the total iron concentration over the concentration range covered, providing an easy control of the synthesis.

Example 4 – *Role of ultrasonication*

The size distributions for the particles formed under various degree of ultrosonication intensity are shown in Figure 9. It is evident that a stronger ultrasonication intensity results in a smaller size product, but levels off at intensity above 25% of the full scale. This finding not only illustrates the critical role of ultrasonication in controlling the particle sizes of the product, but also illustrates the optimal intensity of ultrasonication, i.e., around 30% of the full scale in the present case. It is interesting to note that although the sizes of the synthesized particles is highly dependent on ultrasonication intensity, the magnetization characteristics changes only marginally as shown in Figure 10. This finding tends to suggests that the extent of conversion/crystallization is less sensitive to ultrasonication intensity. The ultrasonication appears to play a dual role in this case: forming smaller reactant drops as it enters mother alkaline solutions and mechanically limiting the aggregation/sintering of precipitates during crystallization.

Example 5 - *Cell Separation*

The magnetic nanoparticle suspensions prepared as such were tested for biological cell separations in a commercial laboratory. The amount of dextran adsorbed on magnetite was determined to be as high as 0.29 g-

dextran/g-solids. The presence of the dextran on the nanoparticles not only acted as a particle stabilizer, but also provided a means for coupling with light density human peripheral blood cells through tetrameric antibody complex (TAC) recognition using well established procedures (Thomas, et al., 1992). The general procedure is outlined below:

(1) Prepare nucleated cell suspension at a concentration of 1 x 10^8 cells/mL in buffered cell culture medium (e.g. PBS + 2% Fetal Bovine Serum +1 mM EDTA). Place cells in a 12 x 75 mm tube.

(2) Add TAC directed against a specific cell surface antigen (e.g. CD14, CD19). Mix well and incubate at room temperature for 15 minutes.

(3) Add the particles at the desired concentration as assessed based on weight fraction of the particle suspension or on the optical density of the particle suspension at a wavelength of 450 nm. Mix well and incubate at room temperature for 10 minutes.

(4) Add buffered cell culture medium such that the cell suspension volume is approximately the same as the active volume of the magnet (the volume where the magnetic field gradient is high). Mix the cells in the tube by gently pipetting up and down 2-3 times. Place the tube in the magnet and wait 5 minutes for the separation to take place.

(5) Pick up the magnet and tube, and in one continuous motion invert the magnet and tube, pouring off the supernatant fraction. The magnetically labeled cells will remain inside the tube, held by the magnetic field of the magnet. Leave the magnet and tube in an inverted position for 2-3 seconds, then return to upright position.

(6) To increase the frequency of magnetically labeled cells relative to the unlabeled cells (the desired cell purity) repeat the separation procedure.

(7) Remove tube from magnet and resuspend cells in an appropriate amount of cell culture medium.

This procedure was applied to the separation of CD19+ and CD14+ cells. The CD19 antigen is associated with B cells and the CD14 antigen is associated with monocytes. B cell separations resulted in a purity of 96% and a recovery of 37% of the CD19+ cells initially present. The monocyte

separations resulted in a purity of 97% with a recovery of 66% of the cells initially present.

The cell separation was effected by a magnetic field gradient generated without the magnetizable medium used in high gradient magnetic separation. This was possible due to the stronger magnetization of the prepared particles relative to other available particles such as Molday (1984). The particle diameter range of 30 to 250 nm allows for diffusible particles which results in effective targeting to the cells. The superparamegnetic nature of the particles, ensures a high stability of the fluids against magnetic flocculation. In addition, because particles in this size range are in a sense invisible to biological systems, the particles can be used for in vivo clinical applications such as vehicles for drug delivery and for nondestructive clinical diagnosis.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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CLAIMS

1. A method of synthesizing superparamagnetic magnetite particles comprising:

(a) controlling the crystallization of magnetite to form superparamagnetic particles by adding a solution of ferric and ferrous ions to an alkaline solution, thereby limiting the duration of ferric and ferrous ion supersaturation and the extent of magnetite crystal growth;

(b) controlling aggregation of said magnetite particles to control the size distribution of said particles; and

(c) coating said particles with a coating substance to generate non-aggregating particles, thereby stabilizing the particle suspension.

2. The method of claim 1 wherein the aggregation is prevented.

3. The method of anyone of claims 1 or 2 wherein the aggregation of particles is controlled by physical means.

4. The method of claim 3 wherein the physical means is sonication.

5. The method of claim 1 wherein the ratio of ferric to ferrous ions is 2:1.

6. The method of claim 1 wherein at least steps (a) and/or (b) are performed in a redox environment to inhibit the oxidation of Fe2+ to Fe3+ iron.

7. The method of claim 6 wherein the redox environment is controlled by nitrogen sparging.

8. The method of claim 1 wherein said solution of ferric and ferrous ions has a pH of about 2.5.

9. The method of claim 1 wherein said alkaline solution is a sodium hydroxide solution.

10. The method of claim 8 wherein said sodium hydroxide solution is a 1.5M sodium hydroxide solution.

11. The method of claim 1 wherein the coating substance is dextran.

12. The method claim 11 wherein said dextran has a molecular weight of about 40,000.

13. The method of claim 11 wherein said dextran solution is a .25M dextran solution.

14. The method of claim 1 wherein the coating substance is selected from the group consiting of: BSA, antibodies, natural or synthetic polymers, protein, polypeptides or nucleic acid polymers.

15. The method of claim 1 wherein the nanoparticles are from 10 to 200 nm in diameter.

16. The method of claim 1 wherein the nanoparticles are 20 to 150 nm in diameter.

17. The magnetite nanoparticles produced by the method of anyone of claims 1-16.

18. The magnetite nanoparticles of claim 17 wherein the particles are superparamagnetic.

19. A method of rendering a biological target magnetizable comprising, functionalizing the magnetite nanoparticles of claim 17 or 18 to enable them to couple with the biological target and then exposing the biological target to the functionalized magnetite nanoparticles to form a nano particle-target complex.

20. The method of claim 19 wherein the nanoparticles are functionalized by derivatizing the surface of the particles putting a specific ligand on the particle.

21. The method of claim 20 wherein the nanoparticles are functionalized by an anti-dextran antibody.

22. The method of claim 19 wherein the biological targets are cells, protein, DNA or RNA.

23. The method of claim 19 to 22 wherein the nanoparticle-target complex is recovered from a sample by magnetic separation.

24. The method of claim 23 wherein magnetic separation is achieved by exposing the sample to an external magnetic field gradient.

Figure 1

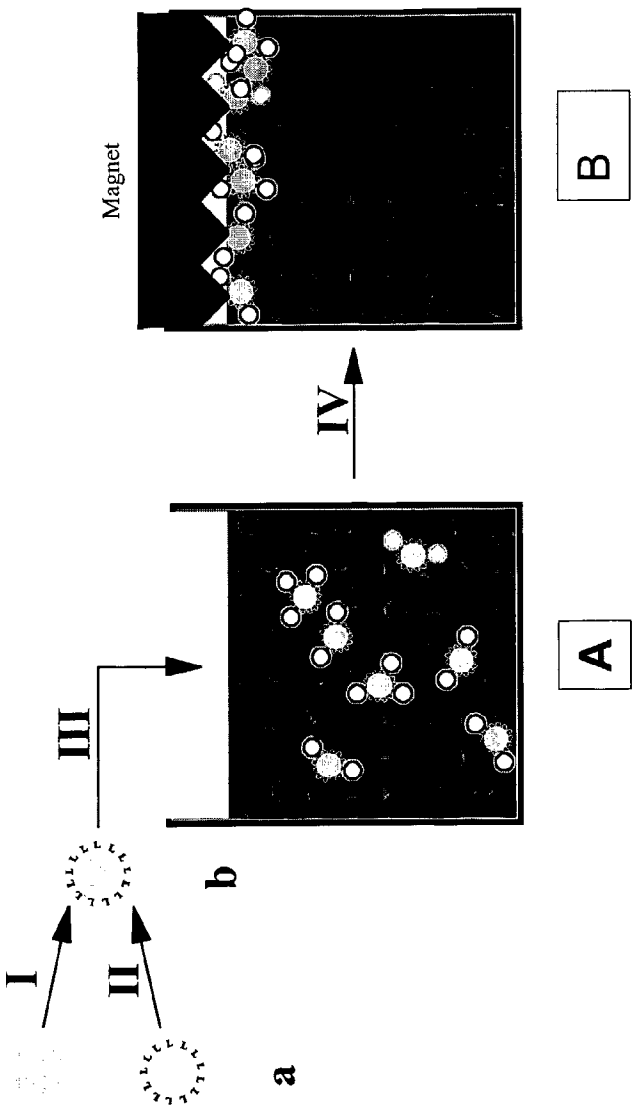

Figure 2

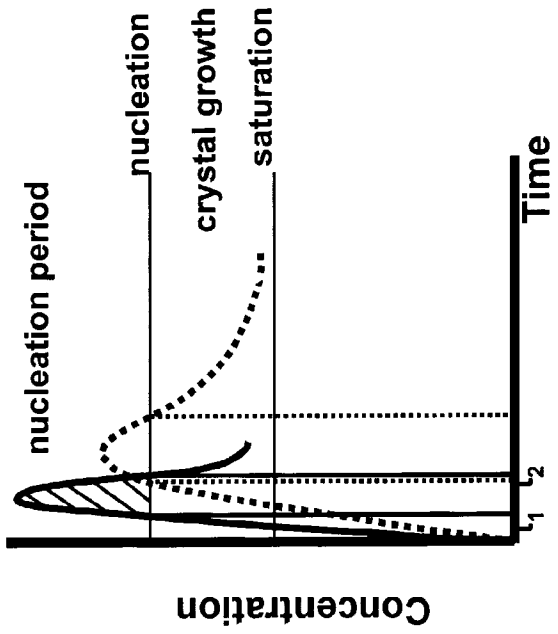
nucleation period
nucleation
crystal growth
saturation
Time
Concentration
2
1

Figure 3

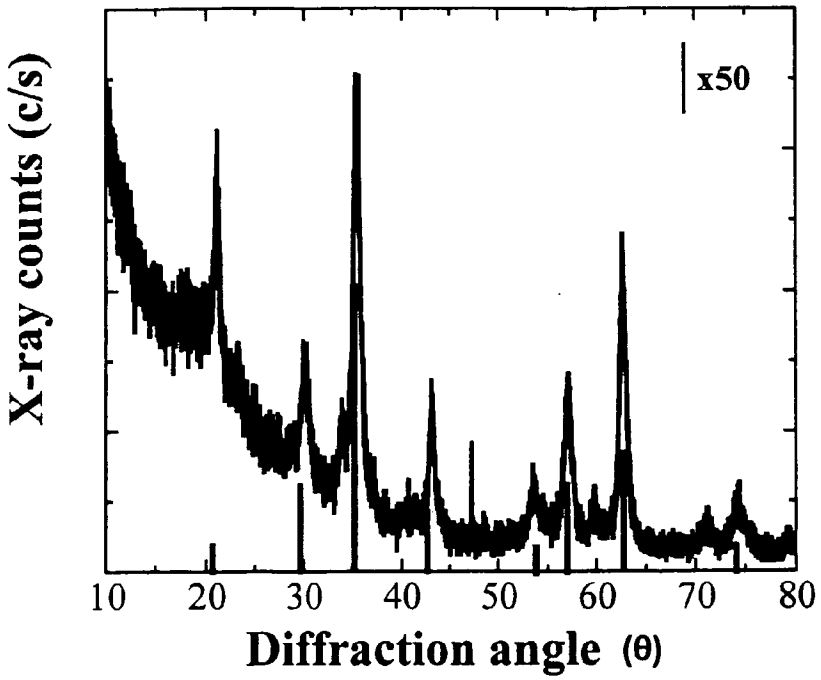

Figure 4

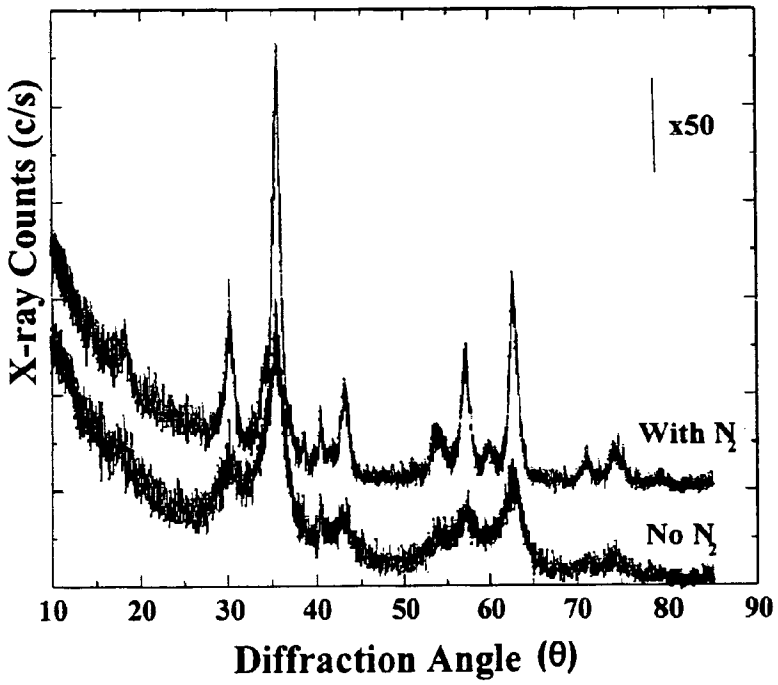
X-ray Counts (c/s)
x50
With N2
No N2
10
20
30
40
50
60
70
80
90
Diffraction Angle (θ)

Figure 5

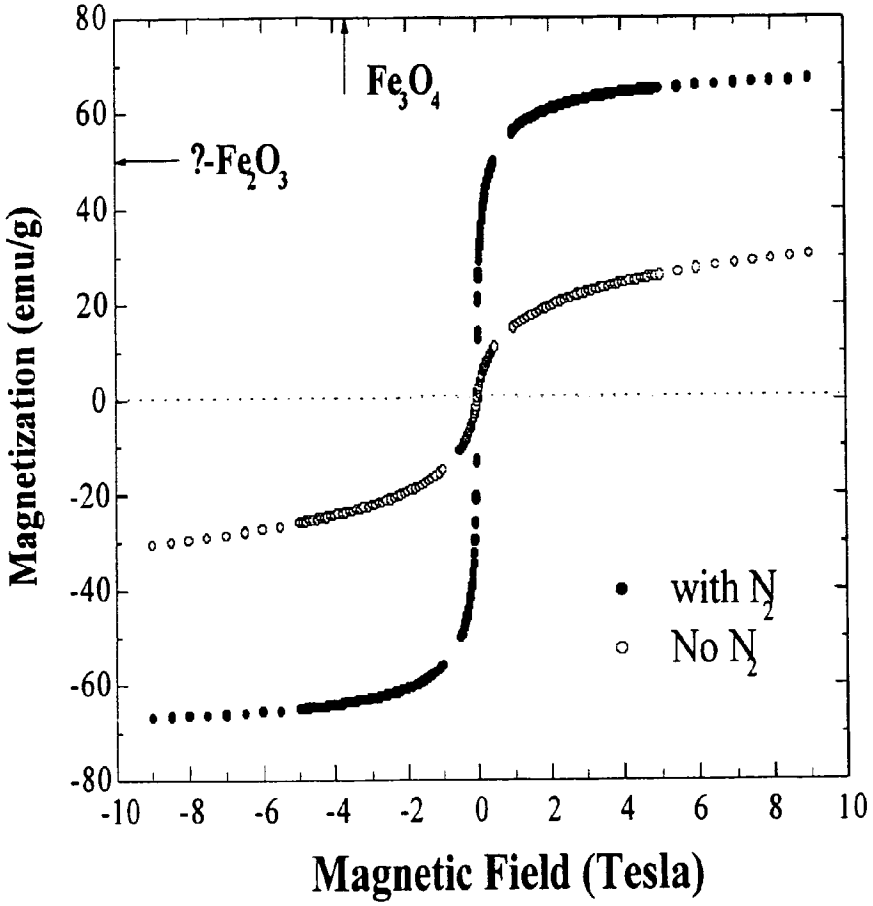
Fe3O4
?-Fe2O3
with N2
No N2
Magnetization (emu/g)
Magnetic Field (Tesla)
80
60
40
20
0
-20
-40
-60
-80
-10
-8
-6
-4
-2
0
2
4
6
8
10

Figure 6

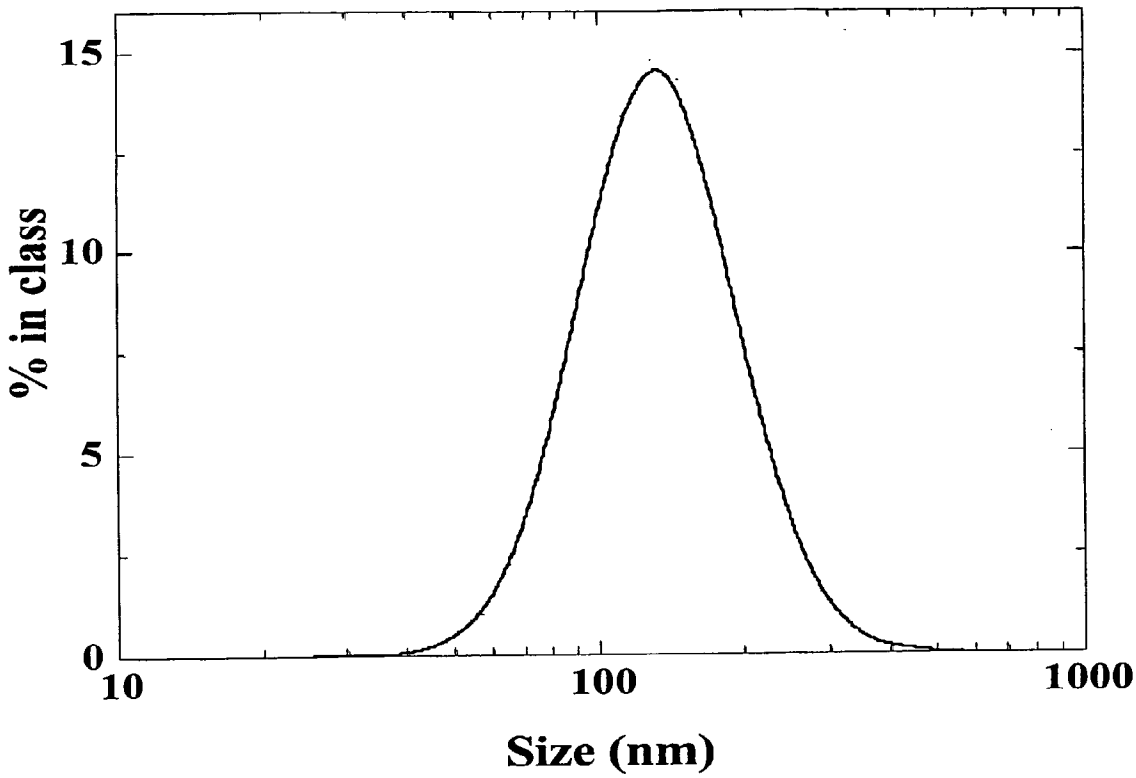

Figure 7

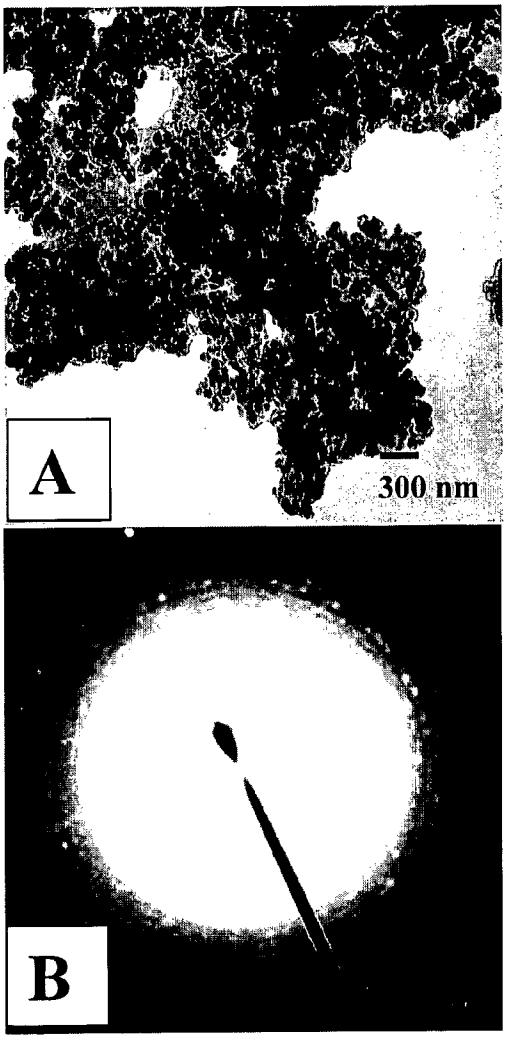

Figure 8

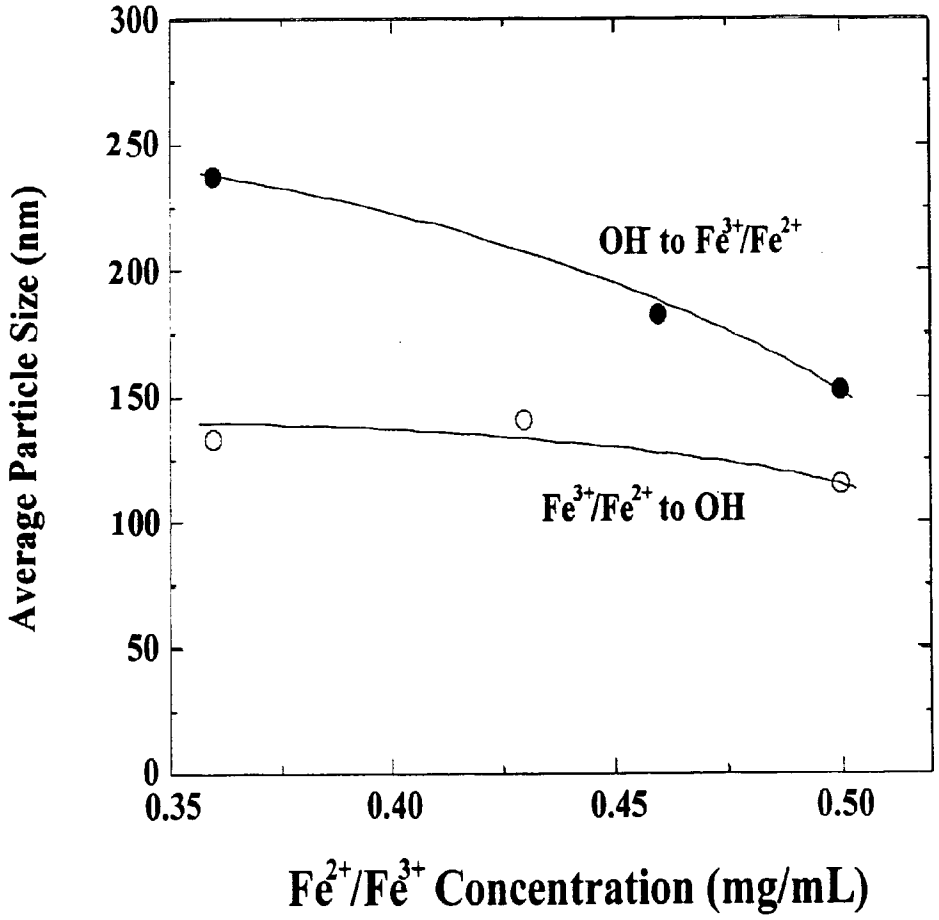
300
250
200
150
100
50
0
0.35
0.40
0.45
0.50
Average Particle Size (nm)
OH to Fe3+/Fe2+
Fe3+/Fe2+ to OH
Fe2+/Fe3+ Concentration (mg/mL)

Figure 9

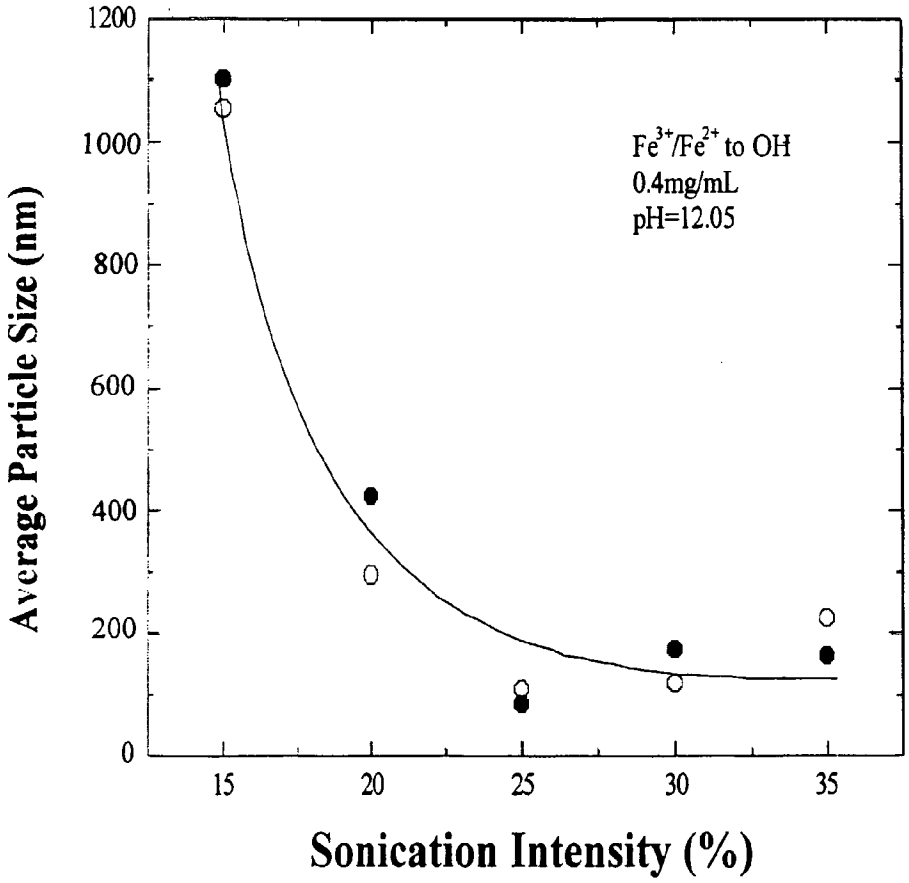
1200
1000
800
600
400
200
0
Average Particle Size (nm)
15
20
25
30
35
Sonication Intensity (%)
Fe3+/Fe2+ to OH
0.4mg/mL
pH=12.05

Figure 10